

## STIC-ILL

**From:** Davis, Minh-Tam  
**Sent:** Thursday, February 03, 2005 1:10 PM  
**To:** STIC-ILL  
**Subject:** Reprint request for 10/057813

10/057813 Adonis  
BIO Tech V. MAIN  
IC NOS  
RECEIVED 2/3/05

1) 10773013 PMID: 10893637  
Induction of apoptosis in BPH stromal cells by adenoviral-mediated overexpression of caspase-7.  
Marcelli M; Shao T C; Li X; Yin H; Marani M; Denner L; Teng B; Cunningham G R  
Departments of Medicine, Baylor College of Medicine and VA Medical Center, Houston, Texas, USA.  
Journal of urology (UNITED STATES) Aug 2000, 164 (2) p518-25,  
ISSN 0022-5347 Journal Code: 0376374

2) ###13937305 PMID: 9634557  
Induction of caspase-3-like protease may mediate delayed neuronal death in the hippocampus after transient cerebral ischemia.  
Chen J; Nagayama T; Jin K; Stetler R A; Zhu R L; Graham S H; Simon R P  
Department of Neurology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213, USA.  
Journal of neuroscience - the official journal of the Society for Neuroscience (UNITED STATES) Jul 1 1998, 18 (13) p4914-28,  
ISSN 0270-6474 Journal Code: 8102140

Thankyou.  
MINH TAM DAVIS  
ART UNIT 1642, ROOM 3A24, MB 3C18  
272-0830

## INDUCTION OF APOPTOSIS IN BPH STROMAL CELLS BY ADENOVIRAL-MEDIATED OVEREXPRESSION OF CASPASE-7

MARCO MARCELLI, T. C. SHAO, XIAOYING LI, HEATHER YIN, MICHELA MARANI,  
LARRY DENNER, BABIE TENG AND GLENN R. CUNNINGHAM\*

From the Departments of Medicine, Baylor College of Medicine and VA Medical Center, the Department of Cell Biology, Texas Biotechnology Corporation, and the Institute of Molecular Medicine, University of Texas Health Science Center, Houston, Texas

### ABSTRACT

**Purpose:** We hypothesized that expression/activity of critical components of the apoptotic pathway can be used to induce apoptosis of a human prostate cell line derived from benign prostatic hyperplasia (BPH) tissue.

**Materials and Methods:** We analyzed the apoptotic pathway in BPH cells treated with the powerful inducer of apoptosis, staurosporine (STS), and adenoviruses overexpressing caspase-3, -7, or the control gene lacZ.

**Results:** Twelve hours post-STS, most BPH cells were floating in the culture medium, TUNEL staining was widespread, and DEVDase activity (the catalytic activity of type II caspases) was increased. The pan-caspase inhibitor, Z-VAD-FMK, prevented STS-induced apoptosis. Based on these observations, we performed immunoblot analysis for the three known group II caspases (that is caspase-2, -3 and -7), but none of them was detected with three commercially available antibodies. Nevertheless, in view of the presence of increased DEVDase activity, we reasoned that a group II caspase must be a critical mediator of apoptosis in this model. If correct, we postulated that overexpression and activation of a type II caspase should cause apoptosis. To test this hypothesis, we coupled the cDNAs encoding caspase-3 and caspase-7 to adenoviral vectors and obtained constructs AvC3 and AvC7. Cells infected with AvC3 or AvC7 overexpressed the protein for caspase-3 or -7 within 24 to 48 hours. Caspase-3 overexpression did not cause apoptosis above that observed in cells receiving the control adenovirus expressing the lacZ cDNA (AvLac-Z). In contrast, caspase-7 overexpression induced massive apoptosis. BPH cells were then infected with increasing multiplicity of infection (MOI) of AvC7 and AvLacZ. A positive correlation was found between the amount of caspase-7 expressed and the level of DEVDase activity measured. AvC7 at MOIs of 25:1 and 50:1 induced apoptosis in about 50% of BPH cells at 72 hours post-infection. This effect was AvC7 specific, because the same MOIs of AvLacZ were not apoptogenic.

**Conclusions:** Adenoviral-mediated overexpression of caspase-7 induces apoptosis of BPH-derived cells.

KEY WORDS: benign prostate hyperplasia, apoptosis, caspases

Benign prostatic hyperplasia (BPH) is a disease associated with aging, and it is primarily a disease of the stroma. The increase in prostate volume seen with BPH is in part related to cellular hyperplasia. Stereologic analysis indicates that the stroma:epithelium volume is increased 33% over that in the normal prostate.<sup>1</sup> These findings have been confirmed by quantitative morphometry in prostates from men with symptomatic BPH.<sup>2</sup> The histological composition of 23 hyperplastic prostates was 74.78 ± 2.29% stromal and 17.72 ± 2.16% epithelium.<sup>3</sup> Using surface markers and immunohistochemistry, BPH stromal nodules consisted of 8.8% immature mesenchymal, 65.2% fibroblastic, 21.6% fibromuscular and 4.4% smooth muscle cells.<sup>4</sup>

Although the increase in stroma could be due to increased mitotic activity, there is evidence that reduction in cell death is a major factor. First, stromal cells have long life spans. Using estimates of superoxide dismutase activity, the average life span of BPH stromal cells has been estimated to be more than 30 years.<sup>5</sup> Second, in a study that used TGFβ as a marker for apoptosis and Bcl-2 as an indicator of anti-

apoptotic activity, TGFβ immunostaining of BPH tissue was localized to the secretory epithelial cells<sup>6</sup> but none was observed in the stroma. Third, a recent study found the mean proliferation index in the epithelium and stroma to be similar.<sup>7</sup> The apoptotic rate in epithelium was similar to the proliferative rate, but no apoptotic cells were detectable in the stroma. These investigators concluded that stromal growth in BPH is due to cell proliferation in the absence of cell death.

Available medical therapies are only modestly effective for mild or moderately symptomatic BPH. 5α-reductase inhibitors have not been demonstrated to significantly increase apoptosis of prostate stromal cells *in vivo*. Alpha-adrenergic inhibitors may cause a transient increase in apoptosis of BPH stromal and epithelial cells.<sup>8</sup> However, this does not appear to be the major mechanism for their *in vivo* effects since prostate volume has not been demonstrated to be reduced.

Because BPH is primarily a disease of the stroma, effective medical therapy for moderate to severe disease will require destruction of these cells. One approach to reduce the number of BPH stromal cells is to activate or overexpress genes involved in the apoptotic pathway.

Many aspects of the basic biology of the apoptotic pathway

Accepted for publication March 3, 2000.

\* Requests for reprints: 2002 Holcombe Blvd., Houston TX 77030.  
Supported by a Merit Review grant from the Department of Veteran's Affairs.

have been elucidated in recent years. The histological features of cells undergoing death by apoptosis include DNA fragmentation, nuclear condensation, membrane blebbing and phosphatidylserine externalization.<sup>9-11</sup> These changes occur following the engagement of two major apoptotic pathways, one triggered by activation of death receptors,<sup>12</sup> and the other by functional incapacitation of the mitochondria and their release to the cytosol of pro-apoptotic factors such as cytochrome c.<sup>13</sup> The final, execution phase of both pathways is mediated by activation of the caspases, a family of cysteine proteases. It has been proposed that initiator caspases with long prodomains, such as caspase-8 or -9, either directly or indirectly activate effector caspases, such as caspase-3 and -7.<sup>14-16</sup> These effector caspases then cleave and inactivate intracellular factors that are critical for cell survival, such as poly(ADP-ribose) polymerase (PARP) and lamins, and cause the typical apoptotic morphology.

Our investigation has sought to study the mitochondrial pathway in BPH stromal cells undergoing apoptosis. Once molecules potentially critical to the programmed cell death pathway of this cell line were identified, we tried to induce apoptosis by manipulating their expression/activity using adenoviral technology.

#### MATERIALS AND METHODS

**Materials.** Staurosporine was purchased from Alexis Corporation (San Diego, CA). Tissue culture reagents were obtained from Life Technologies (Frederick, MD). Molecular biological reagents were obtained from New England Biolab (Beverly MA). A mixture of protease inhibitors (CompleteTM) was provided by Boehringer Mannheim (Indianapolis IN). Z-VAD-FMK and Z-DEVD-AFC were purchased from Enzyme System, Dublin CA. Ac-DEVD-CHO was purchased from Biomol (Plymouth Meeting, PA). All other chemicals were bought from Sigma (St. Louis, MO). We obtained antibodies for caspase-2, -3 and -7 from Transduction Laboratories (Lexington, KY). The enhanced chemiluminescence detection kit was from Amersham Corp. (Arlington Heights, IL). We purchased the Easy DNA kit from Invitrogen, (San Diego, CA), the In Situ Cell Death Detection Kit from Boehringer (Indianapolis, IN) and Slowfade from Molecular Probes, (Eugene, OR). 293 cells were obtained from Microbix Biosystem (Toronto, CA). TUNEL positive cells were scored using a fluorescent microscope (Olympus IX70, Olympus America, Melville, NY). Images were recorded with a digital camera SPOT (Diagnostic Instruments, Sterling Heights, MI).

**Cell culture and experimental design.** BPH stromal cells were isolated from BPH chips obtained at the time of transurethral prostatectomy.<sup>22</sup> Briefly, seared tissue was removed and viable tissue was washed in medium A [Ham's F-12 and Waymouth MD 705/1(1:1 v/v) supplemented with penicillin (200 U/ml.) and streptomycin (200 mg/ml.)]. The tissue was minced and digested with collagenase (5 mg/ml.) in medium A enriched with fetal calf serum (FCS), and rotated in a tube at room temperature for four hours. Tissue was passed through a #18 gauge needle 6 to 7 times and then centrifuged for 10 minutes. The pellets were washed once with medium A, and then were re-suspended in the culture medium. The culture medium consisted of 10% FCS plus medium A, but the concentrations of penicillin and streptomycin were reduced to 100 U/ml. and 100 mg/ml., respectively. Cells were frozen after the 6 to 7<sup>th</sup> passage. The cells were alpha-actin positive, desmin positive, vimentin positive and cytokeratin negative (not shown).

For adenoviral experiments, the stromal cells were grown in Corning 6-well plates until they were approximately 80 to 90% confluent. At the time of infection, the medium was withdrawn from the well. One-half ml of F-12 medium supplemented with 2% FCS and 10  $\mu$ l. of the AvC7 in Tris buffer

containing Mg and 10% glycerol was added into the well. The plate was shaken for 30 to 60 minutes at 37 C in the CO<sub>2</sub> incubator. At the end of incubation, 2 ml. of the medium supplemented with 10% FCS was added.

For staurosporine experiments, 5  $\times$  10<sup>5</sup> cells were seeded in the 6-well plate two days before experiment. On the day of the experiment cells received fresh medium. Staurosporine was dissolved in DMSO and added at varying concentrations. Controls were treated with the same concentration of DMSO. Adherent and floating cells were harvested at times as noted.

**Apoptosis assays: DNA laddering.** Genomic DNA was extracted from adherent and floating cells using the Easy DNA kit. Pure genomic DNA (20  $\mu$ g.) was sized in a 2% agarose gel. We detected apoptosis by the appearance of the typical DNA fragmentation pattern (laddering), indicative of internucleosomal DNA cleavage.<sup>17</sup> To study involvement of the caspases in mediating STS-induced apoptosis, STS was given in association with the caspase inhibitor Z-VAD-FMK (100  $\mu$ M). Prevention of apoptosis was determined by DNA laddering and TUNEL analysis (see below) of cells receiving STS alone or in combination with Z-VAD-FMK.

**TUNEL staining.** Adherent and floating cells were harvested simultaneously, transferred to glass slides using a Cyto-Tek centrifuge (Miles, Elkart, IN), air dried and fixed with 4% formaldehyde in phosphate buffered saline (PBS) for 30 minutes. After washing with PBS, cells were incubated with the TUNEL reaction mixture (Boehringer, Indianapolis, IN) at 37C for 1 hour. Slides were washed 6 times with PBS, mounted in Slowfade, viewed under fluorescence and phase microscopy, and photographed at 50X using a digital camera. Apoptosis was indicated by the appearance of brightly labeled, polyfragmented nuclei and apoptotic bodies.

**Flow cytometry.** Floating and adherent cells were pooled after treatment with trypsin. DNA strand breaks were labeled with biotin-16-dUTP using deoxynucleotidyl exotransferase (TdT). Briefly, we fixed the cells in 1% paraformaldehyde in PBS on ice for 1 hour, washed them with PBS and stored them in 70% alcohol at -20C. The cells were pelleted, washed in cool PBS and decanted. We added fifty  $\mu$ l. of TdT reaction mixture (10  $\mu$ l. 5X reaction buffer, 5  $\mu$ l. CoCl<sub>2</sub>, 0.75  $\mu$ l. TdT, 0.25,  $\mu$ l. biotin-16-dUTP, 34  $\mu$ l. distilled water), and incubated the cells for 1 hour at 37C. After the addition of 1 ml. PBS, cells were centrifuged. The pellets were resuspended in 50  $\mu$ l. of PBS and incubated with 100  $\mu$ l. of Avidin FITC buffer (2.5 mg./ml. Avidin DCS FITC, 4X SSC, 0.1% triton X-100, 5% w/v nonfat dry milk) at room temperature in the dark for 30 minutes. After addition of 1 ml. of 0.1% Triton X-100 in PBS, cells were centrifuged, and the pellet was resuspended in 500  $\mu$ l. of propidium iodide (PI) plus RNAse solution (5  $\mu$ g./ml. of PI 0.1% RNAse in PBS). We determined the cell cycle profile of the PI-stained cells and the percentages of FITC-conjugated apoptotic cells by flow cytometry (Epics Profile Analyzer, Coulter Co., Miami, FL), using AvlacZ infected cells as negative controls. We conducted each treatment in triplicate, and we repeated each experiment at least three times.

**Western analysis.** To study expression of several apoptotic proteins, we used Immunoblot analysis. For the immunodetection of caspase-2, -3, -7, we treated cells with STS for 1 to 24 hours. Floating and adherent cells were pelleted, and the pellet was resuspended in lysis buffer (1% triton-X, 150 mM NaCl, 25 mM Tris pH 7.4, and 8% CompleteTM) and incubated on a rocker for 1 hour at 4C. Cells were centrifuged for 10 minutes in a microcentrifuge, and the supernatant was mixed with loading buffer (45.4 mM Tris pH7.4, 36% glycerol, 11% SDS, 0.05% bromophenol blue, 2% mercaptoethanol) at a ratio of 4:1 (vol:vol) and boiled for 7 minutes at 95C. Aliquots of cell lysates containing 40  $\mu$ g. of proteins were sized by electrophoresis in 12.5% (for caspase-2) or 17% (for caspase-3 and -7) acrylamide gels containing 0.1% sodium doceyl sulfate and transferred to nitrocellulose membrane.

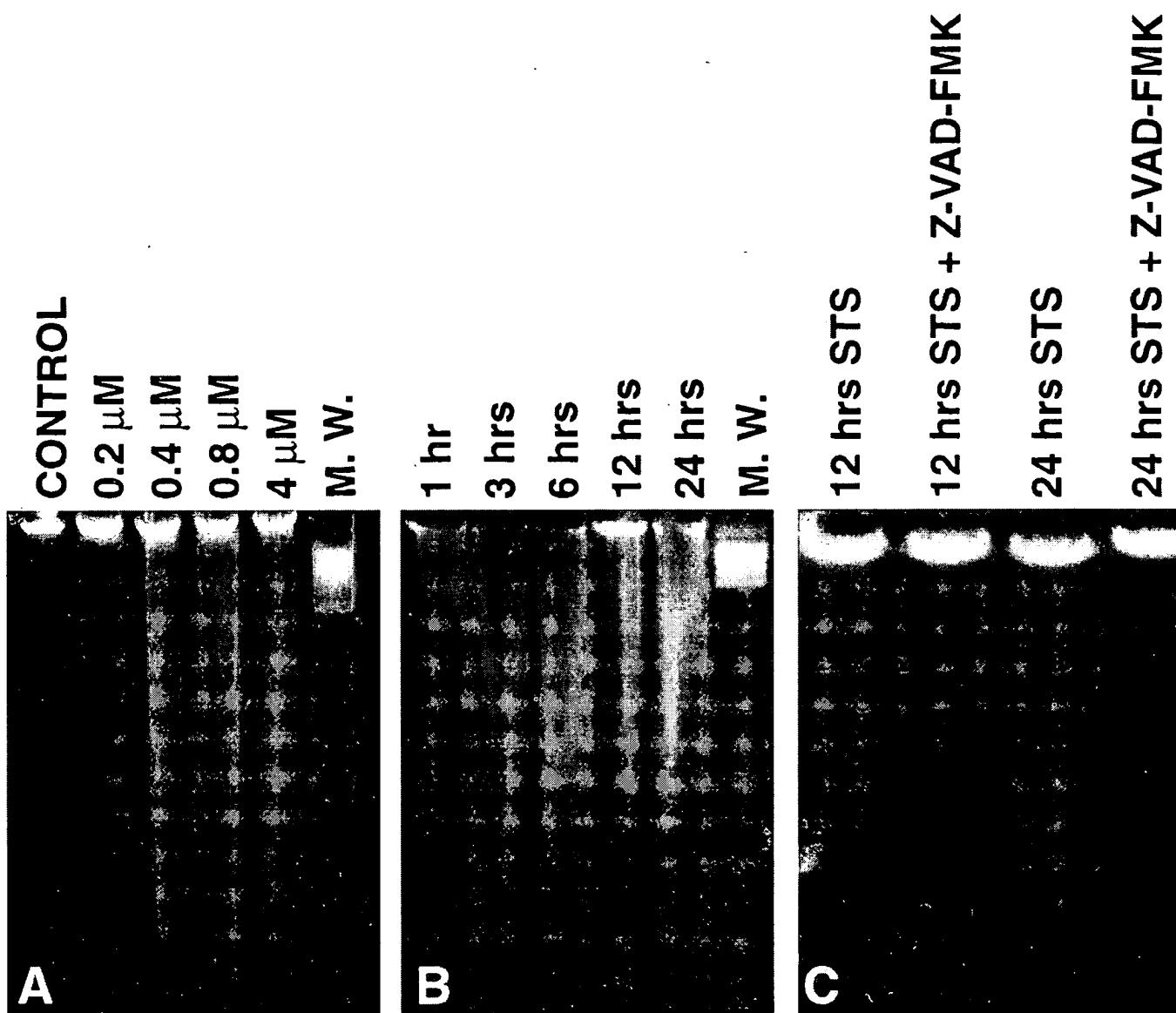


FIG. 1. Staurosporine (STS) induction of apoptosis in BPH stromal cells and Z-VAD-FMK prevention. *A*, cells were treated with STS for 24 hours. *B*, cells were cultured with medium containing 0.8  $\mu$ M STS for indicated time. *C*, cells were cultured in medium containing STS 0.8  $\mu$ M  $\pm$  100  $\mu$ M of pancaspase inhibitor Z-VAD-FMK for 12 or 24 hours. In each case 30  $\mu$ g. of genomic DNA was sized in 2% agarose gels. Each experiment was repeated a minimum of two times.

The membranes were incubated for 60 minutes with antibodies to caspase-2, -3, and -7. After washing, horseradish peroxidase-linked anti-mouse IgG was added and the bands were visualized according to the specifications of the ECL kit manufacturer. The same technique was used to verify caspase overexpression after BPH stromal cells were infected with the viral constructs.

**Assay of DEVDase activity.** DEVDase activity (the catalytic activity of active caspase II<sup>18</sup>) was measured as previously reported.<sup>19,20</sup> Briefly, PBS-washed cell pellets (derived from both adherent and floating cells) were resuspended in extract buffer (25 mM HEPES pH 7.4, 0.1% Triton X-100, 10% glycerol, 5 mM DTT and 1 mM PMSF, 10 mg./ml. pepstatin, 10 mg./ml. leupeptin) and vortexed vigorously. Twenty  $\mu$ l. of extract (corresponding to 10% of the sample) was incubated with the fluorogenic substrate Z-DEVD-AFC at 100  $\mu$ M final concentration for 1 hour at room temperature. The release of AFC was measured by excitation at 400 nm. and emission at 530 nm. on a Cytofluor 2300 fluorescence plate reader using AFC as a standard. In this assay one unit is the amount required to liberate 1 pmol. AFC in 1 hour at 25C using 100

$\mu$ M substrate. The reactions were incubated in the presence or in the absence of equimolar amounts of specific inhibitor (Ac-DEVD-CHO). The specific DEVD cleavage activity was derived by subtracting the amount of fluorescence produced by the samples incubated in the presence of substrate minus the amount released by samples incubated in the presence of substrate plus inhibitor. DEVDase activity was expressed as fold induction versus time point 0 (fig. 2), or as number of DEVDase units/plate (fig. 4 and 7).

**Preparation of adenoviruses.** We made full length caspase-3 and -7 cDNAs by reverse-transcription PCR of total RNA extracted from LNCaP cells, using primers complementary to the published sequences,<sup>21,22</sup> and containing a Kozac's consensus motif before the initial methionine. The products of the amplification contained aminoacids 1-278 and 1-303 of caspase-3 and -7, respectively. The absence of mutations in the resulting constructs was evaluated by sequence analysis.

We made replication-defective recombinant adenoviruses as previously reported.<sup>20,23</sup> Briefly, we subcloned the C3 and C7 cDNA's in vector pAvS6<sup>24</sup> to obtain the shuttle plasmids

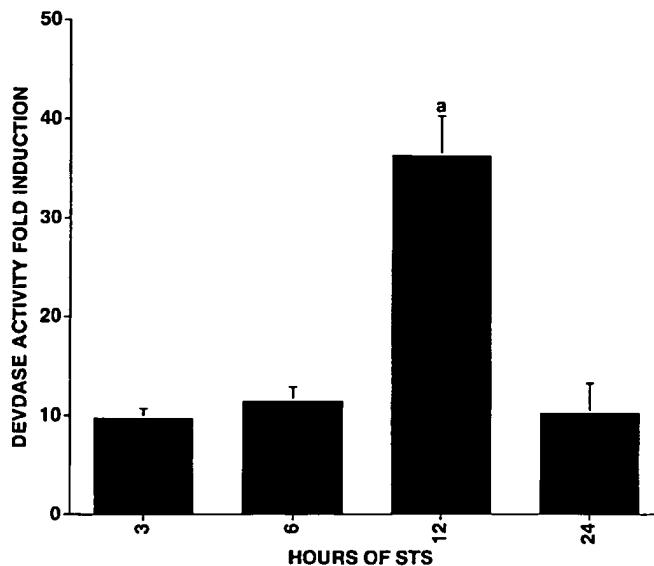


FIG. 2. Induction of DEVdase activity in BPH cells by STS. Cells were treated with 0.8  $\mu$ M STS or vehicle. DEVdase activity was measured as described in Materials and Methods and is expressed as fold-induction versus time-point 0 (mean  $\pm$  SD of three experiments). a indicates significant difference ( $p < 0.001$ ) between time 0 and 3 or 6 hours.

pAvS6-C3 and -C7. pAvS6 has a pBluescript backbone and contains sequences from the left end of the adenovirus 5 genome lacking the early transcription region-1 (E1) that is necessary for replication. In this plasmid the gene of interest is subcloned in a polylinker site located downstream from the Rous sarcoma virus (RSV) promoter and upstream to the SV40 early polyadenylation signal. We cotransfected pAvS6-C3 and C7 with pJM17<sup>25</sup> (that contains a full length adenoviral genome) in low passage 293 cells. This cell line is stably transfected with the SV40 virus, and provides E1 functions in trans. E1-defective recombinant adenovirus (AvC3 and AvC7) was produced by homologous recombination between pAvS6-C3 or pAvS6-C7 and pJM17 in 293 cells. Two weeks after transfection, infectious recombinant adenoviral plaques were picked, expanded, and screened for caspase-3 or -7 sequences by PCR. We purified adenoviral vectors that contained C3 and C7 one more time by plaque assay on 293 cells, and confirmed the correct structure by sequencing and restriction enzyme mapping. Large scale production of high titer recombinant AvC3 and AvC7 was performed by growing 293 cells on improved Eagle's minimum essential medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 50 units/ml. penicillin, 50  $\mu$ g/ml. streptomycin and 1% Fungizone. We purified the viruses twice using cesium chloride density gradient centrifugation. The viral vectors were then dialyzed for  $\geq 8$  h at 4°C against a buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM MgCl<sub>2</sub>, 10% glycerol and stored at -80°C. An aliquot of the two viruses finally was used to determine the viral titer ( $5 \times 10^7$  for AvC3 and  $9 \times 10^7$  for AvC7). We produced recombinant adenovirus AvlacZ, which contains the lacZ cDNA under the control of the RSV promoter, as previously described.<sup>23</sup>

**Infection of BPH cells with viral constructs.** We established in pilot experiments the concentration of control adenovirus AvlacZ infecting 100% of BPH cells without causing apoptosis for a minimum of 4 days. These experiments (data not shown) identified the MOI (multiplicity of infection) of 30 to 50:1 as ideal.  $1 \times 10^6$  BPH cells were seeded on day 0. After 48 hours of growth in regular medium (when the cell population had doubled), cells were infected using varying titers of AvlacZ, AvC3 or AvC7 to achieve MOIs of 0, 5:1, 50:1 and 250:1. The infection was performed by dissolving the neces-

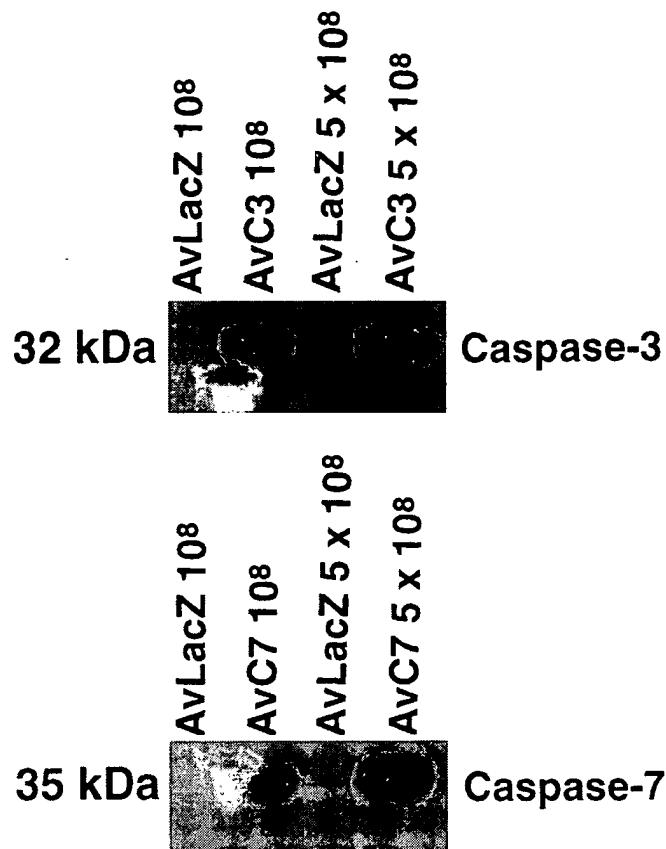


FIG. 3. Adenoviral-mediated overexpression of caspase-3 and -7 in BPH stromal cells. Cells were infected with  $1 \times 10^8$  or  $5 \times 10^8$  pfu of AvLacZ, AvC3 or AvC7 viruses (corresponding to MOIs of 50:1 or 250:1, respectively). After 72 hours they were harvested as described in Materials and Methods and immunoblotted for presence of caspase-3 or -7. Overexpression of two proteins is observed. Each experiment was repeated a minimum of three times.

sary concentration of virus in infection medium (consisting of the medium used to culture each cell line plus 2% FBS and 1% PBS). After adding the appropriate amount of infection medium, each dish was incubated at 37°C in the presence of 5% CO<sub>2</sub> and gently rocked. Normal culture medium was added until the cells were ready for harvesting. Cells were harvested at 24 hours intervals for the following 96 hours and analyzed for the presence of apoptosis, using flow-cytometry and TUNEL. Cells also were analyzed for their ability to overexpress the caspase of interest by immunoblot analysis, and for DEVdase activity. In subsequent experiments BPH cells were infected with AvC7 and AvlacZ at MOIs of 0, 5:1, 25:1, 50:1 and 250:1, and the amounts of protein DEVdase activity and TUNEL positivity at 72 hours post-infection were correlated with the various MOI's.

**Statistical analysis.** Data were analyzed using a two-tailed Student's *t* test and analysis of variance.

## RESULTS

**Induction of apoptosis with staurosporine.** To identify which components of the death pathway were activated in BPH cells undergoing programmed cell death, we used staurosporine (STS) as a powerful and mechanistically pleotropic inducer of apoptosis.  $1 \times 10^6$  BPH cells were incubated with varying concentrations of STS (0.2 to 4  $\mu$ M) for 24 hours. Genomic DNA extracted from each plate exhibited typical apoptotic ladders at each concentration (fig. 1, A). We chose the concentration of 0.8  $\mu$ M for further experiments, based on its ability to produce a pattern of DNA laddering similar to

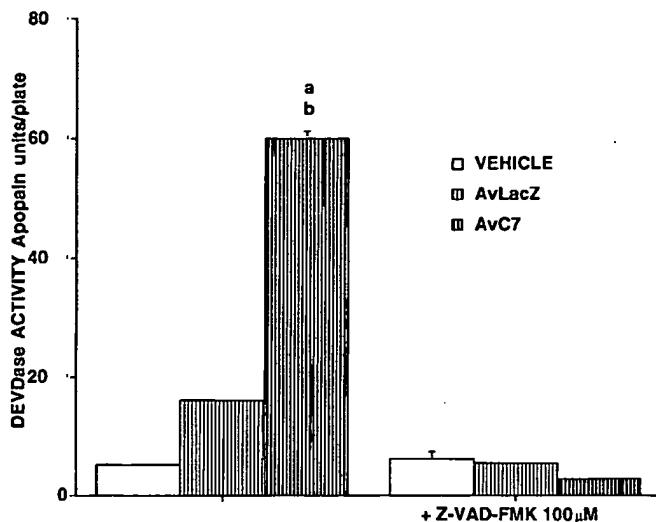


FIG. 4. DEVDase activity measured in BPH cells after infection with AvC7 and AvLacZ (MOI 50:1). DEVDase activity was measured at 24 hours post-infection in the presence or absence of 100  $\mu$ M Z-VAD-FMK as described in Materials and Methods. Each measurement represents mean  $\pm$  SD of three experiments and is expressed as DEVDase units/plate. a indicates significant difference ( $p < 0.001$ ) between cells infected with AvC7 and AvLacZ or vehicle. b indicates significant difference ( $p < 0.001$ ) between cells treated with AvC7 and AvC7 + Z-VAD-FMK.

that induced by the highest concentration of STS (4  $\mu$ M). Using 0.8  $\mu$ M STS, we observed DNA laddering after only 1 hour of treatment (fig. 1, B). At subsequent time-points there was almost no high molecular weight, intact genomic DNA in the cell lysate. After 24 hours 65% of the cells were TUNEL positive in comparison to 4% in the control dish (data not shown).

**DEVDase activity.** We documented caspase activation by incubating the fluorogenic peptide Ac-DEVD-AFC with cell lysates of BPH cells treated with STS. DEVDase activity, which indicates activation of group II caspases (that is caspase-2, -3 and -7),<sup>18</sup> was robustly induced and peaked after 12 hours of treatment with STS (fig. 2). In some experiments STS was given in the presence of the caspase inhibitor z-VAD-FMK. As shown in fig. 1, C, this compound effectively prevented STS-induced apoptosis, an observation that confirms the central role played by group II caspases in mediating STS-induced apoptosis.

**Caspase-2, -3 and -7.** To identify the group II caspase(s) involved in STS-induced apoptosis of BPH cells, we performed immunoblot analysis for the currently known group II caspases<sup>18</sup> (caspase-2, -3 and -7) using cell lysates obtained at various times after addition of STS to the medium. We were unable to detect caspase-2, -3 and -7 using the three commercially available antibodies (see Materials) (data not shown).

**Adenoviral-mediated overexpression of caspase-7.** Based on the previous observations, we postulated that a critical role in STS-induced apoptosis of stromal cells is mediated by an unidentified group II caspase. Because the final aim of our investigation was to detect components of the apoptotic pathway whose manipulation causes apoptosis in BPH cells, and plasmid-induced overexpression of many caspases is associated with induction of apoptosis of the host cell,<sup>26-29</sup> we tested if overexpression of two well-known group II caspases induced apoptosis in this cell line. Based on this, we constructed two adenovirus vectors (AvC3 and AvC7) driven by the constitutively active Rous sarcoma virus (RSV) promoter to achieve overexpression of caspase-3 and -7 in BPH stromal cells.

Both AvC3 and AvC7 induced robust overexpression of their respective proteins (fig. 3). We detected significant

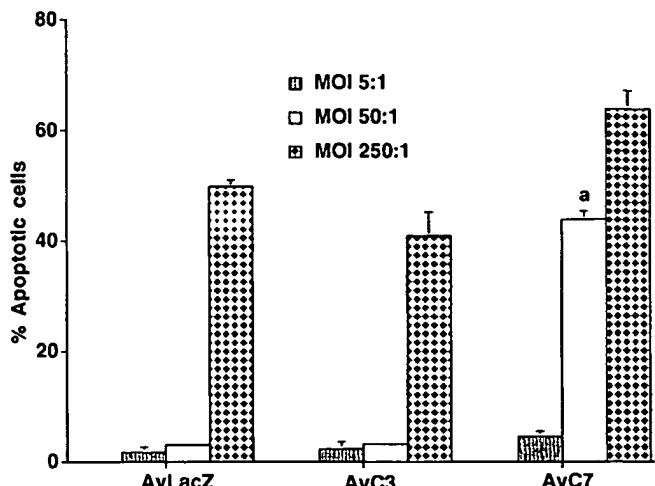


FIG. 5. Percent BPH stromal cells undergoing apoptosis after infection with adenoviruses AvLacZ, AvC3 and AvC7. Cells were seeded on day 0, infected on day 2 using MOIs of 5:1, 50:1 and 250:1 or mock-infected (control), and harvested on day 5. Apoptosis was detected by flow cytometry, as specified in Materials and Methods. No apoptosis was detected in mock-infected cells (not shown). Each time point represents mean  $\pm$  SD of three experiments. a indicates significant difference ( $p < 0.001$ ) between cells infected with AvC7 and AvLacZ or AvC3 at MOI of 50:1.

DEVDase activity in cells infected with a MOI of AvC7 of 50:1 (fig. 4), but not in cells infected with the same concentration of AvC3 (not shown). No significant induction of DEVDase activity was identified in cells infected with AvLacZ.

We conducted a dose response study with each construct using adenovirus containing Lac Z cDNA (AvLacZ) as control. Apoptosis was assessed by labeling apoptotic cells by TUNEL and quantitating the number of apoptotic cells by flow cytometry. BPH stromal cells were cultured for 72 hours in media containing each of the constructs. At a MOI of 5:1 apoptosis was not increased by any of the constructs (fig. 5). Apoptosis was present in 50% of the cells treated with AvC7 at a MOI of 50:1. At this titer apoptosis was not present in the cells treated with AvLacZ or AvC3. Treatment with the high-

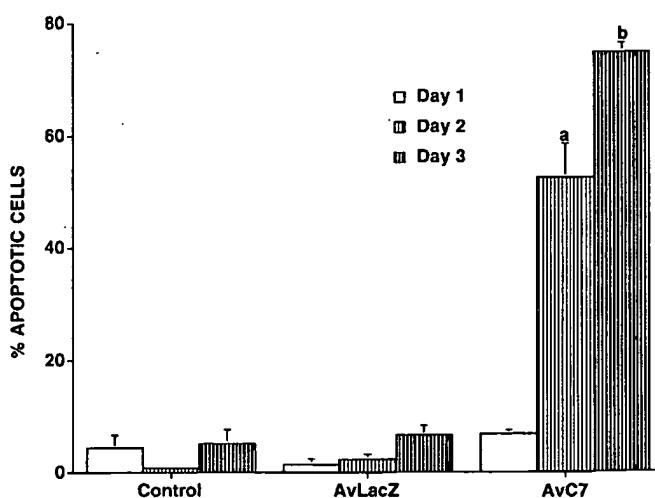


FIG. 6. Apoptosis of BPH cells after 1, 2 and 3 days. Cells were infected with AvLacZ and AvC7 at MOI of 50:1, and harvested on day 1, 2 and 3. Apoptosis was detected by flow cytometry, as specified in Materials and Methods. Each time point represents mean  $\pm$  SD of three experiments. Controls are mock-infected BPH cells. a indicates significant difference ( $p < 0.001$ ) between cells infected with AvC7 and AvLacZ after 2 days of treatment. b indicates significant difference ( $p < 0.001$ ) between AvC7-infected cells and control or AvLacZ-treated cells after 3 days of treatment.

est pfu MOI (250:1) caused apoptosis of cells treated with AvLacZ, AvC3 and AvC7. Thus, at this titer apoptosis was not due to a specific effect of caspase-7, but to intrinsic toxicity of the viral constructs. To assess the optimum time of exposure to the viral construct, we cultured BPH stromal cells for 1 to 3 days in media containing vehicle, AvLacZ or AvC7 (MOI 50:1). Significant apoptosis of BPH stromal cells treated with AvC7 was observed on day 2, and 3 (fig. 6). To further analyze this response, overexpression of caspase-7, induction of DEVDase activity and appearance of TUNEL positivity were correlated in an experiment in which increasing MOIs of AvC7 and AvLacZ (0, 5:1, 25:1, 50:1 and 250:1) were administered to BPH cells for 72 hours. As shown in fig. 7, A and C, increasing MOI of AvC7 (but not of AvLacZ [fig. 7, B]) induced a parallel increase in caspase-7 protein expression. This was followed by increasing induction of DEVDase activity (fig. 7, D). Interestingly, at a MOI of 250:1 there was significant induction of DEVDase activity in AvLacZ-infected cells (fig. 7, E). Approximately 50% of cells were TUNEL positive at MOIs of 25:1 to 250:1 in AvC7-infected cells (8, A, B and C). In contrast, TUNEL positivity was evident in

AvLacZ-infected cells only at a MOI of 250:1 (8, A, D and E). Thus, MOIs of 25:1 and 50:1 (but not 250:1) induced apoptosis in a caspase-7 dependent way. The data presented in fig. 7, D and E suggest that induction of a DEVDase activity of at least 40 Units (obtained with AvC7 at MOIs of 25:1 to 250:1 and AvLacZ at a MOI of 250:1) is sufficient to induce significant apoptosis in BPH cells. This apoptogenic DEVDase activity was triggered by caspase-7 overexpression in BPH cells receiving AvC7. In contrast, it only was triggered by the high titer in cells infected with AvLacZ, presumably due to intrinsic viral toxicity.

#### DISCUSSION

Apoptosis was induced in BPH stromal cells by infecting them with a replication-defective adenovirus containing the cDNA encoding caspase-7. We were encouraged to undertake development of this construct after observing that staurosporine induced apoptosis and DEVDase activity of these cells, and the pancaspase inhibitor Z-VAD-FMK prevented the increase in DEVDase activity and apoptosis. Since in-

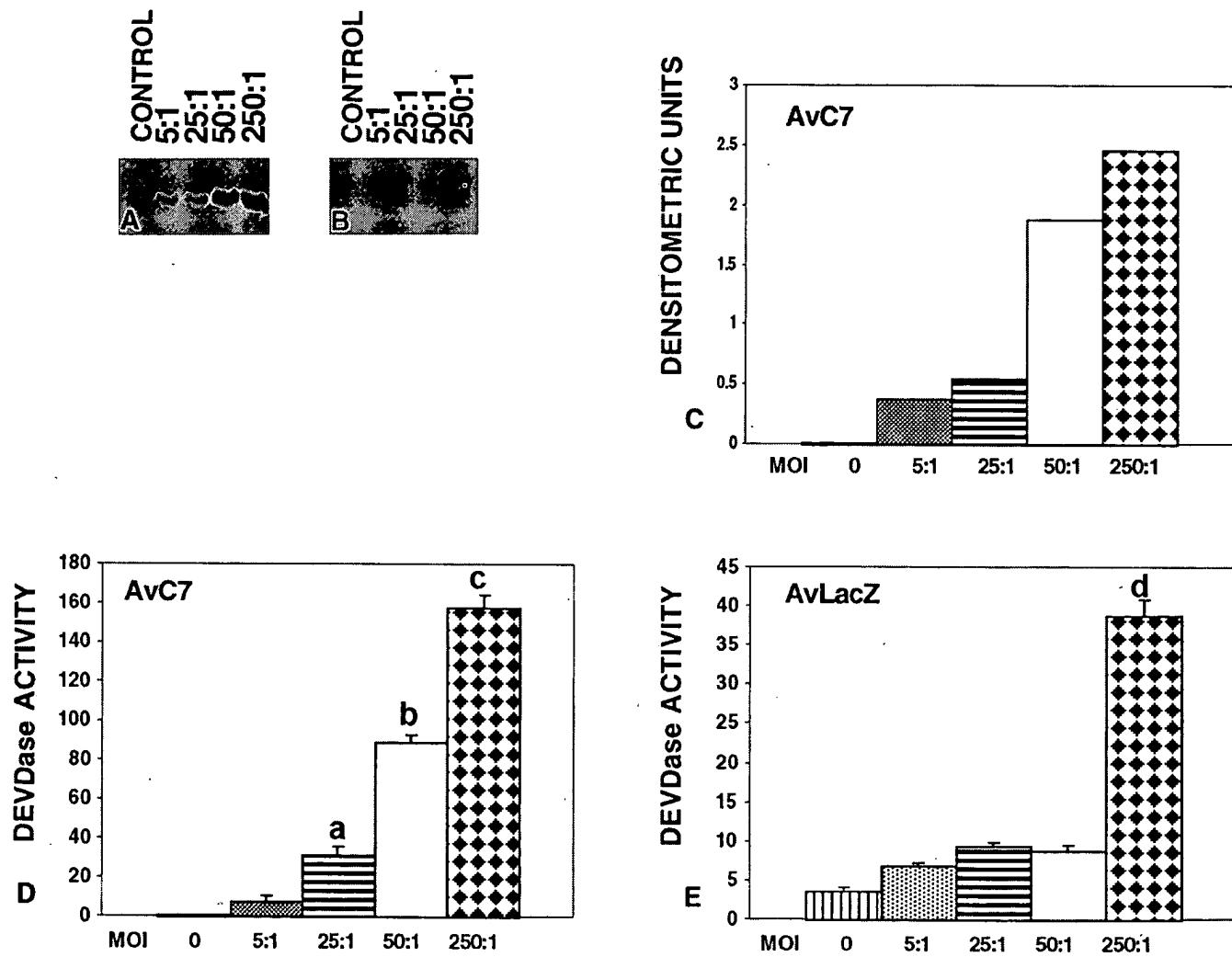


FIG. 7. Correlation of MOI, caspase-7 protein expression and DEVDase activity. A, B, immunoblot analysis of BPH cells infected with increasing MOIs of AvC7 (A) or AvLacZ (B). Increasing MOIs of AvC7 (but not AvLacZ) caused increased overexpression of caspase-7 protein. C, densitometric analysis of Western analysis presented in panel A, showing parallel increase of caspase-7 expression and MOI used. D, E, cells were treated with varying MOIs of AvC7 (D) and AvLacZ (E), harvested and analyzed for DEVDase activity as specified in Materials and Methods. DEVDase activity is expressed as Units of DEVDase/plate. Experiments shown in D (AvC7) and E (AvLacZ) were performed in triplicate and are expressed as mean  $\pm$  SD. a indicates significant difference ( $p < 0.01$ ) between cells treated with AvC7 and AvLacZ at MOIs of 25:1. b indicates significant difference ( $p < 0.001$ ) between cells treated with AvC7 and AvLacZ at MOIs of 50:1. c indicates significant difference ( $p < 0.001$ ) between cells treated with AvLacZ at MOIs of 250:1 and 50:1 ( $p < 0.001$ ). d (panel E) indicates significant difference between cells treated with AvLacZ at a MOI of 250:1 and 0 ( $p < 0.001$ ).

## APOPTOSIS IN BPH STROMAL CELLS

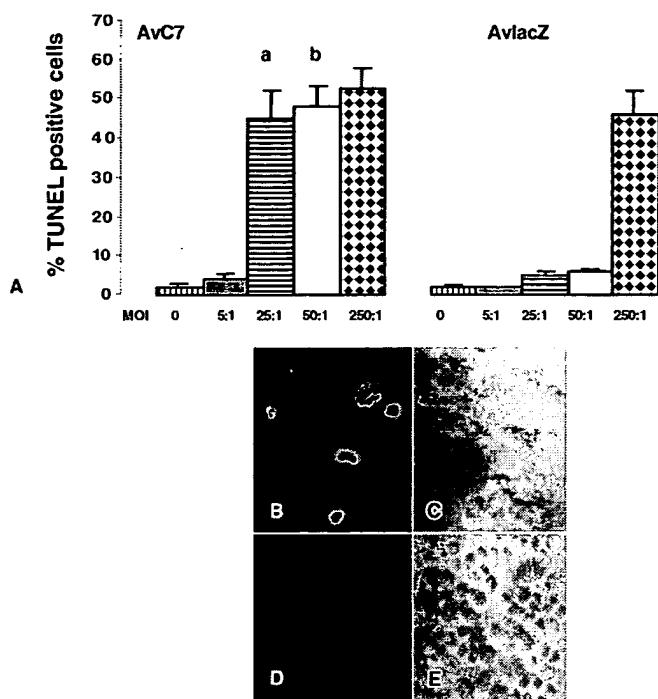


FIG. 8. TUNEL positive BPH cells after infection with AvC7 or AvlacZ. At MOIs of 25:1 to 50:1 there was significant TUNEL positivity in AvC7 (A, B and C) but not AvlacZ (A, D and E)-infected cells. a indicates significant difference ( $p < 0.001$ ) between cells treated with AvC7 and AvlacZ at MOI of 25:1. b indicates significant difference ( $p < 0.001$ ) between cells treated with AvC7 and AvlacZ at MOI of 50:1. At MOI of 250:1 there was similar induction of TUNEL positivity in cells infected with both viruses. B, digital photograph of TUNEL positive AvC7-infected BPH cells (MOI 25:1). D, representative field of BPH cells infected with AvlacZ (MOI 25:1). C, E, corresponding phase contrast microphotographs of AvC7 and AvlacZ-infected cells. As demonstrated by much higher cellularity of specimen, AvlacZ did not induce death at this MOI.

creased DEVDase activity is known to be specific for group II caspases, we used Western analysis to determine which of the known group II caspases was responsible. However, this analysis failed to detect any of the known group II caspases. We reasoned that it was likely that a group II caspase was involved and sought to test this possibility by overexpressing two of the known caspases of this group (-3 and -7) by incorporating their cDNA into adenoviral vectors. When BPH stromal cells were infected with each of these vectors, the cells expressed the appropriate protein, and the AvC7, but not the AvC3, construct induced apoptosis.

The caspase pathway plays a critical role in inducing apoptosis of BPH stromal cells. Our efforts to identify the caspase(s) mediating STS-induced apoptosis among the group II caspases were not successful since caspase-2, -3 and -7 were not immunodetected by three commercially available antibodies. Because these antibodies have been used with success in other cell lines<sup>19,20</sup> and in this cell line after infection with AvC3 and AvC7, a likely interpretation for these findings is that the molecule mediating STS-induced apoptosis in BPH stromal cells is a still unidentified member of the group II caspases. Alternatively, it could be that expression of the known group II caspases in BPH stromal cells is below the threshold for identification by these antibodies. The latter possibility is supported by the work of Krajewska et al, who observed caspase-3 staining in the stromal compartment of the prostate using another antibody.<sup>30</sup>

Plasmid-induced ectopic overexpression of several caspases is followed by apoptotic death of the target cell.<sup>26-29</sup> These molecules express a baseline catalytic activity. It is believed that upon overexpression the baseline catalytic ac-

tivity is amplified, allowing autoactivation of the overexpressed molecule and execution of the downstream apoptotic pathway.<sup>31</sup> However, this was not observed following adenoviral-induced overexpression of caspase-3. A previous report demonstrated that ectopic overexpression of caspase-3 is followed by apoptosis of the host cells only if one uses a chimeric caspase-3 molecule in which the order of the various subunits is rearranged.<sup>29</sup> It is conceivable that caspase-3 is not a good substrate to undergo activation following overexpression. Thus, BPH stromal cells did not undergo apoptosis when they were infected with AvC3.

In contrast, overexpression of caspase-7 caused autocatalytic activation, induction of DEVDase activity, and significant apoptosis of BPH stromal cells. The ability of caspase-7 to induce apoptosis following adenoviral-mediated overexpression is in agreement with other experiments that we have conducted with prostate cancer cell lines.<sup>20</sup> Thus, caspase-7 represents a good substrate that apparently undergoes autoactivation following overexpression, leading to activation of the apoptotic pathway and cell death.

The prostate is the site of two common diseases in aging men, benign prostatic hyperplasia and prostate cancer. Patients affected by advanced BPH usually undergo surgery to improve their obstructive symptoms. Although BPH is a benign disease, considerable morbidity is associated with it. Thus, we conducted the experiments discussed in this paper to study the feasibility of a new gene therapy approach for treating BPH. The data presented show that therapeutic apoptosis of a cell line derived from BPH is achievable using adenoviral-mediated caspase-7 overexpression.

Two prototypes of gene therapy protocols have evolved for the treatment of hyperplastic or neoplastic diseases. When a defective gene causes the disease of interest, it can be replaced by a wild type copy that is inserted into the viral vector. Alternatively, a gene causing cell death can be introduced into a hyperplastic or neoplastic tissue. Use of the herpes simplex virus-thymidine kinase (HSV-TK) cDNA is the most common approach for performing cytoreductive gene therapy. Activation of TK-mediated cytotoxicity occurs following exposure of the cell of interest to gancyclovir (GCV). The use of genes like caspase-7 represents an evolution from HSV-TK, since they are cytotoxic without requiring exposure to substances like GCV. Based on the results presented in this paper and in previous experiments with prostate cancer cell lines,<sup>20</sup> we think that the main issue with the use of apoptotic genes is not whether they will cause cell death, but rather how can they be best targeted to specific tissues. It is predictable that death genes such as caspase-7 will induce a suicidal response in every tissue in which they are concentrated. This is especially true if they are driven, as in our studies, by a constitutively active viral promoter. Therapeutic agents such as drugs, enzymes or viral vectors can be introduced into the prostate by ultrasound guided probes. Alternatively, it may be possible to utilize prostate specific promoters to target these viral vectors. Thus, one of the main issues is to identify prostate specific promoters that restrict transduction of the caspase of interest to the prostate. Promoters specific to prostate epithelial cells have been used successfully to create transgenic models of prostate cancer<sup>32-34</sup> and to direct gene expression to prostate cancer cell lines.<sup>35</sup> Studies are being conducted with these promoters to target vectors *in vivo* to the prostate. While it may be more difficult to identify promoters that are specific for prostate stroma, unpublished studies by D. Rowley (Cell Biology, Baylor College of Medicine, Houston TX) indicate that this may be possible.

Overall, the studies presented in this paper have proven that the intrinsic apoptotic pathway can be used for the purpose of inducing therapeutic apoptosis in cells derived from the stromal compartment of a hyperplastic human prostate. Future experiments will determine if apoptosis can be

induced in the intact prostate of animals where the vascular supply and host defense mechanisms may limit the effects of exogenously introduced apoptotic genes.

We thank Dr. Jack Thornby (VA Medical Center, Houston TX) for help with the statistical analysis.

## REFERENCES

1. Rohr, H. P. and Bartsch, G.: Human benign prostatic hyperplasia: a stromal disease? New perspectives by quantitative morphology. *Urology*, **16**: 625, 1980
2. Shapiro, E., Becich, M. J., Hartanto, V. and Lepor, H.: The relative proportion of stromal and epithelial hyperplasia is related to the development of symptomatic benign prostate hyperplasia. *J Urol*, **147**: 1293, 1992
3. Ishigooka, M., Hayami, S., Suzuki, Y., Hashimoto, T., Sasagawa, I. and Nakada, T.: Age-related changes of histological composition in established benign prostatic hyperplasia. *Eur Urol*, **29**: 85, 1996
4. Bierhoff, E., Walljasper, U., Hofmann, D., Vogel, J., Wernert, N. and Pfeifer, U.: Morphological analogies of fetal prostate stroma and stromal nodules in BPH. *Prostate*, **31**: 234, 1997
5. Tunn, S., Nass, R., Ekkernkamp, A., Schulze, H. and Krieg, M.: Evaluation of average life span of epithelial and stromal cells of human prostate by superoxide dismutase activity. *Prostate*, **15**: 263, 1989
6. Kyrianiou, N., Tu, H. and Jacobs, S. C.: Apoptotic versus proliferative activities in human benign prostatic hyperplasia. *Hum Pathol*, **27**: 668, 1996
7. Claus, S., Berges, R., Senge, T. and Schulze, H.: Cell kinetic in epithelium and stroma of benign prostatic hyperplasia [see comments]. *J Urol*, **158**: 217-21, 1997
8. Kyrianiou, N., Litvak, J. P., Borkowski, A., Alexander, R. and Jacobs, S. C.: Induction of prostate apoptosis by doxazosin in benign prostatic hyperplasia. *J Urol*, **159**: 1810, 1998
9. Kerr, J. F., Wyllie, A. H. and Currie, A. R.: Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer*, **26**: 239, 1972
10. Wyllie, A. H., Kerr, J. F. and Currie, A. R.: Cell death: the significance of apoptosis. *Int Rev Cytol*, **68**: 251, 1980
11. Martin, S. J., Reutelingsperger, C. P., McGahon, A. J., Rader, J. A., van Schie, R. C., LaFace, D. M. and Green, D. R.: Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med*, **182**: 1545, 1995
12. Ashkenazi, A. and Dixit, V.: Death receptors: signaling and modulation. *Science*, **281**: 1305, 1998
13. Liu, X., Kim, C., Yang, J., Jemmerson, R. and Wang, X.: Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell*, **86**: 147, 1996
14. Cohen, G. M.: Caspases: the executioners of apoptosis. *Biochem J*, **326**: 1, 1997
15. Fraser, A. and Evan, G.: A license to kill. *Cell*, **85**: 781, 1996
16. Srinivasula, S. M., Ahmad, M., Fernandes-Alnemri, T., Litwack, G. and Alnemri, E. S.: Molecular ordering of the Fas-apoptotic pathway: the Fas/APO-1 protease Mch5 is a CrmA-inhibitable protease that activates multiple Ced-3/ICE-like cysteine proteases. *Proc Natl Acad Sci USA*, **93**: 14486, 1996
17. Wyllie, A.: Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature*, **284**: 555, 1980
18. Thornberry, N. A., Rano, T. A., Peterson, E. P., Rasper, D. M., Timkey, T., Garcia-Calvo, M., Houtzager, V. M., Nordstrom, P. A., Roy, S., Vaillancourt, J. P., Chapman, K. T. and Nicholson, D. W.: A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. *J Biol Chem*, **272**: 17907, 1997
19. Marcelli, M., Cunningham, G., Haidacher, S., Padayatty, S., Sturgis, L., Kagan, C. and Denner, L.: Caspase-7 is activated during lovastatin-induced apoptosis of the prostate cancer cell line LNCaP. *Cancer Res*, **58**: 76, 1998
20. Marcelli, M., Cunningham, G., Walkup, M., He, Z., Sturgis, L., Kagan, C., Mannucci, R., Nicoletti, I., Teng, B. and Denner, L.: Signaling pathway activated during apoptosis of the prostate cancer cell line LNCaP: overexpression of caspase-7 as a new gene therapy strategy for the treatment of prostate cancer. *Cancer Res*, **59**: 398, 1999
21. Fernandes-Alnemri, T., Litwack, G. and Alnemri, E.: CPP32, a novel human apoptotic protein with homology to *C. elegans* cell death protein Ced-3 and mammalian interleukin-1 $\beta$ -converting enzyme. *J Biol Chem*, **269**: 30761, 1994
22. Fernandes-Alnemri, T., Takahashi, A., Armstrong, R., Krebs, J., Lawrence, F., Tomaselli, K., Wang, L., Yu, Z., Croce, C., Salvesen, G., Earnshaw, W., Litwack, G. and Alnemri, E.: Mch3, a novel human apoptotic cysteine protease highly related to CPP32. *Cancer Res*, **55**: 6045, 1995
23. Teng, B., Blumenthal, S., Forte, T., Navatnam, N., Scott, J., Gotto, A. and Chan, L.: Adenovirus-mediated gene transfer of rat apolipoprotein B mRNA-editing protein in mice virtually eliminates apolipoprotein B-100 and normal low density lipoprotein production. *J Biol Chem*, **269**: 29395, 1994
24. Smith, T., Mehaffey, M., Kayda, D., Saunders, J., Yei, S., Trapnell, B., McClellan, A. and Kaleko, M.: Adenovirus-mediated expression of therapeutic plasma levels of human factor IX in mice. *Nature Genet*, **5**: 397, 1993
25. McGrory, W., Bautista, D. and Graham, F.: A simple technique for the rescue of early region 1 mutations into infectious human adenovirus type 5. *Virology*, **163**: 614, 1988
26. Miura, M., Zhu, H., Rotello, R., Hartwieg, E. and Yuan, J.: Induction of apoptosis in fibroblasts by IL-1 $\beta$ -converting enzyme, a mammalian homolog of the *C. elegans* cell death gene ced-3. *Cell*, **75**: 653, 1993
27. Wang, L., Miura, M., Bergeron, L., Zhu, H. and Yuan, J.: Ich-1, an Ice/ced-3-related gene, encodes both positive and negative regulators of programmed cell death. *Cell*, **78**: 739, 1994
28. Duan, H., Orth, K., Chinmayaian, A., Poirier, G., Froelich, C., He, W.-W. and Dixit, V.: ICE-LAP6, a novel member of the ICE/Ced-3 gene family, is activated by the cytotoxic T cell protease granzyme B. *J Biol Chem*, **271**: 16720, 1996
29. Srinivasula, S., Ahmad, M., MacFarlane, M., Huang, Z. L., Fernandez-Alnemri, T. and Alnemri, E.: Generation of constitutively active recombinant caspases-3 and -6 by rearrangement of their subunit. *J Biol Chem*, **273**: 10107, 1998
30. Krajewska, M., Wang, H. G., Krajewski, S., Zapata, J. M., Shabaik, A., Gascoyne, R. and Reed, J. C.: Immunohistochemical analysis of in vivo patterns of expression of CPP32 (Caspase-3), a cell death protease. *Cancer Res*, **57**: 1605, 1997
31. Vaux, D. and Strasser, A.: The molecular biology of apoptosis. *Proc Natl Acad Sci USA*, **93**: 2239, 1996
32. Greenberg, N., DeMayo, F., Sheppard, P., Barrios, R., Lebovitz, R., Finegold, M., Angelopoulou, R., Dodd, J., Rosen, M. and Matusik, R.: The rat probasin gene promoter directs hormonally- and developmentally-regulated expression of a heterologous gene specifically to the prostate in transgenic mice. *Mol Endocrinol*, **8**: 230, 1994
33. Wei, C., Willis, R. A., Tilton, B. R., Looney, R. J., Lord, E. M., Barth, R. K. and Frelinger, J. G.: Tissue-specific expression of the human prostate-specific antigen gene in transgenic mice: implications for tolerance and immunotherapy. *Proc Natl Acad Sci USA*, **94**: 6369, 1997
34. Cleutjens, K. B., van der Korput, H. A., van Eekelen, C. C., van Rooij, H. C., Faber, P. W., and Trapman, J. An androgen response element in a far upstream enhancer region is essential for high, androgen-regulated activity of the prostate-specific antigen promoter. *Mol Endocrinol*, **11**: 148, 1997
35. Gotoh, A., Ko, S.-C., Shirakawa, T., Cheon, J., Kao, C., Miyamoto, T., Gardner, T., Ho, L.-J., Cleutjens, C., Trapman, J., Graham, F. and Chung, L.: Development of a prostate-specific antigen promoter-base gene therapy for androgen-independent human prostate cancer. *J Urol*, **160**: 220, 1998